Betty A. and Donald J. Baumann Family Scholarship Fund Application Form

1. **Name and NetID**
   Tyler Woodward (tjw32971)

2. **Chemistry faculty research director**
   Dr. Benjamin Brandsen

3. **Proposal title**
   Cell-free Biosynthesis to Generate New Lasso Peptides

4. **Proposal description. Please limit the proposal to about 500 words and include figures as appropriate. Your proposal should briefly outline the overall project and its goal(s). If you have previous results related to your proposed project, concisely summarize those results and describe what you expect to accomplish during the time frame of the scholarship.**

Proposal

With the rise of antibiotic resistance, a robust strategy of developing new antibiotics is crucial to combat the growing threat. One exciting new sources of antibiotics are ribosomally-synthesized, post-translationally modified peptides (RiPPs). Lasso peptides, a family within RiPPs, are small peptides known for their lariat structure which some have been identified as having antibacterial properties, targeting the RNA polymerase. Furthermore, lasso peptides are attractive therapeutics because of their thermostability, resistance to proteases, and bioengineering potential. Klebsidin, identified from *Klebsiella pneumoniae*, is a 19 amino acid lasso peptide that exhibits narrow antibacterial activity toward *K. pneumoniae*.

Previously, I analyzed single amino acid substitutions of klebsidin and their effect on functionality. Additionally, I have designed and examined chimeric klebsidin peptides which incorporated substitution mutations modeling other lasso peptides. Both of these projects demonstrated the high tolerance to mutation of klebsidin at certain positions. While these prior projects utilized cell-based expression, there are limitations to producing highly mutated variants using cell-based expression. Figure 1 shows bacterial growth for insertion variants in the loop region. The lower bacterial growth for variants 8 and 11 suggests inefficient export from the cell, which leads to inhibition of cellular growth, and suggests cellular expression of highly mutated klebsidin variants may not be feasible. This problem could significantly hinder our efforts to use RiPP biosynthesis as potential antibiotics. Therefore, I sought to develop a method that decouples klebsidin expression from cellular export and cellular growth.

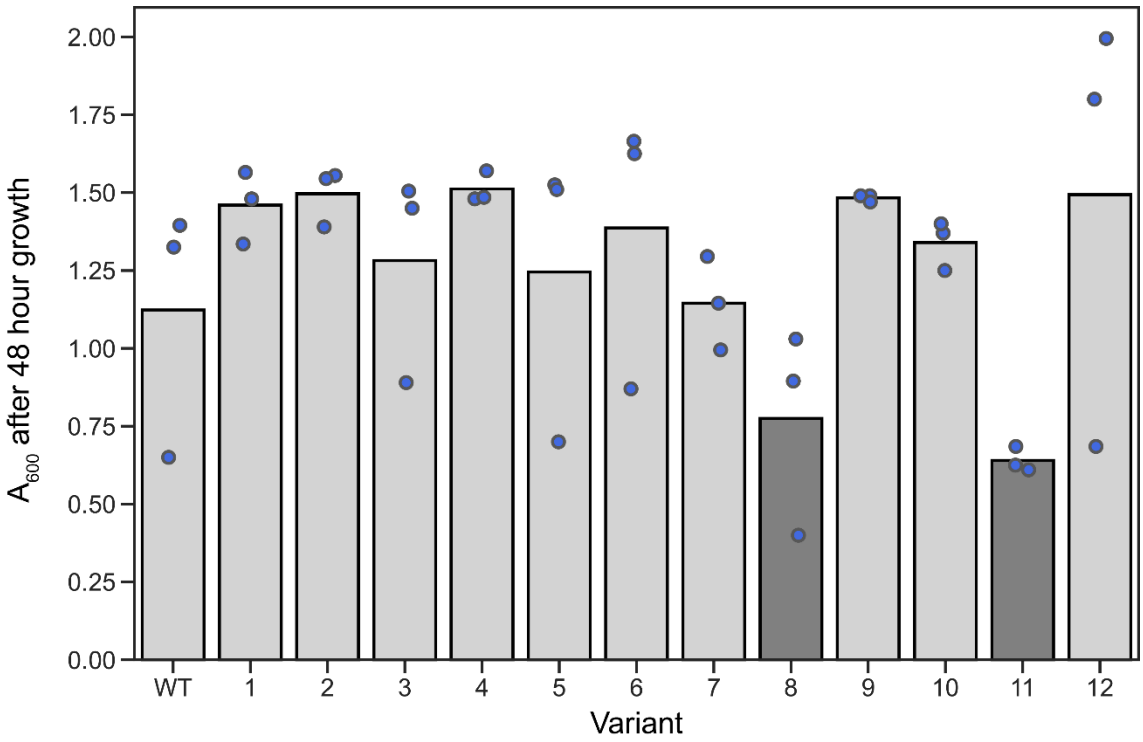

Figure 1. Growth of bacteria with plasmids encoding the klebsidin biosynthetic pathway. Each plasmid encoded a unique klebsidin variant with an insertion in the peptide loop region. After 48 hours of growth, optical density was assessed using spectrophotometer. Variants 8 and 11 showed reduced growth compared to WT klebsidin and other variants suggesting inefficient export of the lasso peptide.

One strategy I am exploring to decouple lasso peptide expression from export and cellular growth is using cell-free expression (CFE). Cell-free expression (CFE) takes advantage of the in vitro activity of T7 RNA polymerase and the ribosome to produce peptides and proteins from plasmid DNA in a plastic microcentrifuge tube. Furthermore, CFE utilizes a T7 RNA polymerase identified from a bacteriophage which is not susceptible to inhibition from klebsidin. Over the past summer, I successfully optimized CFE conditions by measuring expression of a fluorescent reporter gene. Additionally, I successfully produced using CFE wild type klebsidin, as well as several chimeric lasso peptides derived from klebsidin, and analyzed them using MALDI-TOF mass spectrometry.

The project I propose seeks to produce lasso peptides that contain additional posttranslational modifications using tailoring enzymes found in other RiPP biosynthetic pathways using CFE. This is a strategy to increase the chemical diversity of lasso peptides and introduce new functional groups not encoded by the twenty proteinogenic amino acids. I have chosen three promiscuous tailoring enzymes from other biosynthesis pathways that can be utilized including a epimerase, a cyclodehydratase, and a lanthipeptide synthetase. First, I will design plasmids encoding these klebsidin variants and additional modification enzymes that are compatible with the CFE expression platform. Second, I will evaluate CFE reactions utilizing these plasmids utilizing the optimized conditions found for expressing wild type klebsidin, evaluating the products from each reaction using mass spectrometry and reverse-phase high-performance liquid chromatography. Finally, I will conduct activity assays to characterize the import of these variants into cells and their potential as new antibiotics. If successful, this work

will aid in our understanding of the best strategies to engineer RiPP biosynthetic pathways and expand the diversity of known lasso peptides.

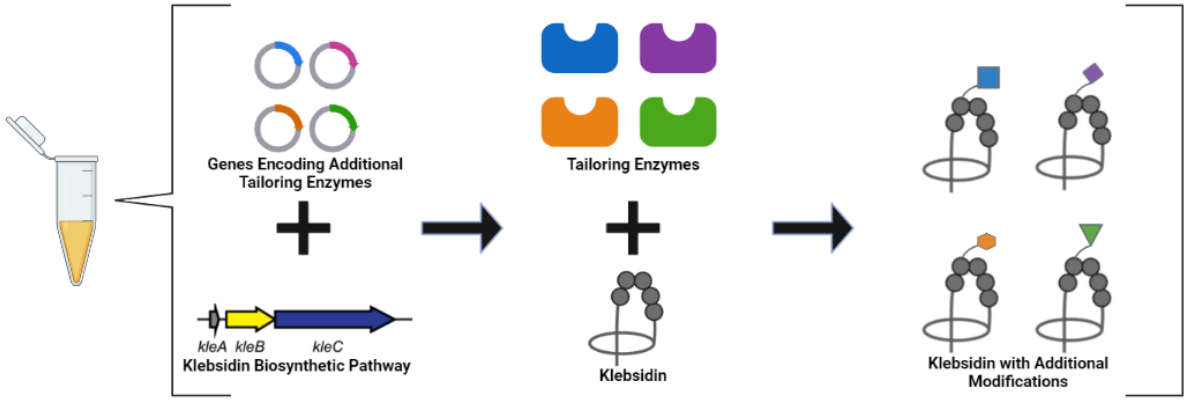

Figure 2. Proposed strategy for using CFE to make new lasso peptides. This project seeks to install modifications from other tailoring enzymes known to be promiscuous. The CFE method will be carried out in a microcentrifuge tube at room temperature, and peptide products will be directly analyzed from the CFE reaction.

References

(1) Sun, Z. Z., Hayes, C. A., Shin, J., Caschera, F., Murray, R. M., & Noireaux, V. (2013). Protocols for implementing an Escherichia coli based TX-TL cell-free expression system for synthetic biology. *Journal of Visualized Experiments*, (79), e50762. https://doi.org/10.3791/50762

(2) D. Silverman, A., Kelley-Loughnane, N., B. Lucks, J., & C. Jewett, M. (2019). Deconstructing Cell-Free Extract Preparation for in Vitro Activation of Transcriptional Genetic Circuitry. *ACS Synthetic Biology*, *8*(2), 403–414. https://doi.org/10.1021/acssynbio.8b00430

(3) Bogart, J. W., Cabezas, M. D., Vögeli, B., Wong, D. A., Karim, A. S., Jewett, M. C. (2021). Cell-Free Exploration of the Natural Prdouct Chemical Space. *ChemBioChem*, (22), 84-91. https://doi.org/10.1002/cbic.202000452

(4) Si, Y., M. Kretsch, A., M. Daigh, L., J. Burk, M., & A. Mitchell, D. (2021). Cell-Free Biosynthesis to Evaluate Lasso Peptide Formation and Enzyme–Substrate Tolerance. *Journal of the American Chemical Society*, *143*(15), 5917–5927. https://doi.org/10.1021/jacs.1c01452

5. Presentation of research results (past and future conferences, publications, seminars, etc.)

Past presentations

Creighton CURAS Research and Scholarship Fair (March 2021)
Creighton University Research Week (April 2021)
Nebraska Academy of Sciences (April 2021)
ACS Midwest Regional Meeting (October 2021)

Future presentations

Nebraska Academy of Sciences (Spring 2022)

Creighton University Research Week (Spring 2022)
National Council on Undergraduate Research (April 2022)

6. **Post-graduate plans (job market, graduate school, medical school, etc.)**
   Graduate School to pursue a Ph.D. in biochemistry beginning Fall 2022.

7. **Number of semesters involved in research, including current semester (summers count as two semesters)**
   9 Semesters

8. **Anticipated graduation date**
   May 2022